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Isolation and Molecular Characterization Of Antimicrobial Agent Producing Bacteria Isolated From Palandoken Mountain

Mustafa Özkan BALTACI[1]

[1] Atatürk Üniversitesi, Fen Fakültesi, Moleküler Biyoloji ve Genetik Bölümü, Erzurum, Türkiye
Mustafa Özkan BALTACI ORCID No: 0000-0003-4968-9016

**Sorumlu yazar: ozkanbaltaci@atauni.edu.tr*

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Abstract: *Streptomyces* is a genus of Gram-positive bacteria that grows in different habitats, and its shape takes after filamentous fungi. The most effective characteristic of *Streptomyces* is the ability to produce secondary metabolites, such as antivirals, antifungals, anti-hypertensives, antitumorals, and especially antibiotics. In this study, bacteria producing antimicrobial substances were isolated from soil samples collected from Palandöken Mountain. Test strains were identified using conventional (morphological, physiological and biochemical tests) and molecular methods (16S rRNA sequencing). Then, the antagonistic effect of these bacteria against pathogenic microorganisms was determined by disc diffusion method. As a result of analysis, it was found that two bacteria (AO1 and AO3) were similar to *Streptomyces violaceochromogenes*, one (AO2) to *Streptomyces ambofaciens* and other (AO4) to *Sphingomonas melonis* at a rate of 99%. According to conventional tests, all isolates were catalase positive, three were oxidase negative (except AO4). In addition, pH, NaCl and temperature values that isolates can growth were determined. Also, phylogenetic trees of the isolates were performed by the neighbor-joining method. Finally, the antimicrobial properties of bacteria were investigated. It was determined that the isolates generally showed high antimicrobial effect against *Escherichia coli* O157: H7 strain and the lowest antimicrobial effect was shown against *Staphylococcus aureus* strain.

Palandöken Dağı'ndan İzole Edilen, Antimikrobiyal Ajan Üreten Bakterilerin İzolasyonu Ve Moleküler Karakterizasyonu

Anahtar kelimeler
Antimikrobiyal maddeler, *Streptomyces*, Moleküler karakterizasyon, Polifazik yaklaşım

Öz: *Streptomyces*ler, farklı habitatlarda gelişen bir Gram-pozitif bakteri cinsi olup, şekli ipliksi mantarlara benzer. *Streptomyces*'in en etkili özelliği antiviraller, antifungaller, anti-hipertansifler, antitümoraller ve özellikle antibiyotikler gibi sekonder metabolitler üretebilmesidir. Bu çalışmada, test suşları, geleneksel (morfolojik, fizyolojik ve biyokimyasal testler) ve moleküler yöntemler (16S rRNA sekanslama) kullanılarak tanımlandı. Daha sonra bu bakterilerin patojen mikroorganizmalara karşı antagonistik etkisi disk difüzyon yöntemi ile belirlendi. Analiz sonucunda, izole edilen dört bakteriden, ikisinin (AO1 ve AO3) *Streptomyces violaceochromogenes'e*, birinin (AO2) *Streptomyces ambofaciens'*e ve diğerinin ise (AO4) *Sphingomonas melonis'*e % 99 oranında benzer olduğu bulundu. Geleneksel testlere göre, tüm izolatlar katalaz pozitif, üçü oksidaz negatiftir (AO4 hariç). Daha sonra izolatların büyüyebileceği pH, NaCl ve sıcaklık değerleri belirlendi. Ardından izolatların filogenetik ağaçları komşu birleştirme yöntemi ile yapılmıştır. Son olarak bakterilerin antimikrobiyal özellikleri araştırıldı. İzolatlar genel olarak *Escherichia coli* O157:H7 suşuna karşı yüksek antimikrobiyal etki gösterdiği ve en düşük antimikrobiyal etkinin *Staphylococcus aureus* şusuna karşı gösterildiği tespit edildi.

1. INTRODUCTION

Antibiotics are substances that are usually synthesized by living microorganisms such as some bacteria and fungi, have a stopping or lethal effect on many microorganisms and are mostly used in the treatment of infectious diseases [1]. Especially in recent years, although the research and development activities of the

international pharmaceutical industry have increased every year, it is reported that there is a serious decrease in the number of newly discovered and patented drugs. Therefore, the antimicrobial activity of many *Streptomyces* species is important for the pharmaceutical industry. Antibiotics commonly found in nature; They play a regulatory role in the microbial population of soil, water, sewage and compost. In recent years, the emergence of many strains of pathogenic microorganisms has developed resistance to antibiotics, which poses a serious threat to public health. The truth is that some pathogenic microorganisms are resistant to all antibiotics in existence and cannot be cured. Therefore, completely new types of antibiotics are needed. Due to the resistance of pathogenic microorganisms to antibiotics, the treatment of infectious diseases is getting more difficult and material and moral loss in such diseases has been increasing day by day [2-4]. Increasing number of antibiotic-resistant strains due to misuse of antibiotics has led us to find new antibiotic compounds [5, 6]. Modern medicine has managed to overcome many life-threatening diseases, but the threat of antibiotic-resistant bacteria seems to be a never-ending challenge facing humanity. If no action is taken, by 2050 the death toll from antibiotic-resistant strains will be higher than deaths caused by cancer [7]. This dangerous situation, in which the wrong use of antibiotics is seen as the biggest reason, is tried to be overcome by various methods [8]. One of these methods is to find and synthesize new antimicrobial substances. As is known, molds and bacteria produce antibiotics [9]. The vast majority of microbial diversity (> 95%) is still undiscovered. It is clear that such an area that stands before us and is waiting to be discovered has great potential [10]. The regions where transportation is difficult and the human population is low where the chances of finding new species are higher. Isolation, identification and molecular characterization of bacteria producing antimicrobial metabolites from soil samples taken from summit of Palandöken mountain were performed in present study. Then their antagonistic effects on pathogenic microorganisms were determined.

In the light of all this information, our aim in this study, the molecular characterization and antimicrobial properties of bacteria isolated from Palandöken mountain were determined for the first time.

2. MATERIALS AND METHODS

2.1. Collection of Samples

Soil samples from different heights of Erzurum, Palandöken Mountain, from a height of approximately 3000 meters, were collected in sterile containers and stored at room temperature until they were studied.

2.2. Isolation of Bacteria

1 gram of soil sample was placed in falcon tubes containing 50 ml sterile 0.9% NaCl. A serial dilution (dilution series from 100 to 10-7) was prepared. 100 µl of these dilutions were taken and spread on TSA agar plates. These petri dishes were left to incubate for 48 hours at 37 °C under aerobic conditions. Finally, the colonies formed on the petri dish were selected and their pure cultures were prepared [11]. It was determined that four out of twenty six bacteria isolated produced antimicrobial substances.

2.3. Morphological Physiological and Biochemical Characteristics of Bacteria

Oxidase reagent (Sigma 70439) was used for testing oxidase activity. Catalase activity was observed by the production of bubbles after the addition of a drop of 3 % (v/v) H2O2. Gram-reaction was carried out following the method performed by Adiguzel et al [12]. In order to determine temperature range for growth, test strains were grown in Tryptic Soy Broth (TSB) at 15, 20, 25, 30, 37, 40, 45, 50, and 55°C for 48 h. Growth at various NaCl concentrations (0–10 %, at intervals of 1.0 %) was determined in TSB medium. The pH range for growth was determined (4.0–11.0 at 0.5 unit intervals) in TSB medium All parameters (temperature, salt concentration and pH value) were performed in triplicate and measured at OD600 nm [13].

2.4. Molecular Characterization of Bacteria

DNA isolation of strains were performed according to Wizard® Genomic DNA Purification Kit protocol. Briefly, for DNA isolation. the cell walls of the isolates were first lysed with lysozyme. Incubate at 37°C for 30–60 minutes. Samples were centrifuged at 13000 rpm and the supernatant discarded. Then 600 µl of Nuclei Lysis Solution, 3µl of RNase Solution and 200µl of Protein Precipitation Solution was added and centrifuged at 13000 rpm. The supernatant was transferred to tubes containing 600 µl isopropanol and centrifuged. Later, 70% ethanol was added to wash the DNA. Last DNA was dissolved in rehydration solution. The 16S rRNA region, was amplified using UNI16S-L: (5'-ATTCTAGAGTTTGATCATGGCTCA-3') and UNI16S-R: (5'-ATGGTACCGTGTGACGGGCGG TGTGTA-3') primers. 30 µL volume of PCR mixture containing, 17.1 µl ddH2O, 3 µl 10X PCR buffer, 1.2 µl DMSO, 0.6 µl dNTP, 1.8 µl MgCl2, 1.5 µl (10 µM) reverse primer, 1.5 µl (10 µM) forward primer, 3 µl Taq DNA polymerase and 3 µl template DNA [12]. PCR programme was given in Table 1. The amplified fragments were cloned into Escherichia coli JM101 strain with the pGEM-T Easy Cloning Vector (Promega, Southampton, UK) according to protocol of the manufacturer. After the cloning stage, plasmid isolation was carried out. The colonies which gave the positive result, were sequenced by the Macrogen Company (Netherlands). 16S rRNA sequence was compared with the other bacterial strains in the GenBank and EzTaxon (http://blast.ncbi.nlm.nih. and http://www.eztaxon.org), the similarity rate between them was designated. Considering the results of the study, a phylogenetic tree was constructed neighbor-joining method using the software package MEGA 4.0 [14].

Table 1. 16S rRNA PCR steps

	Temperature (°C)	Time (min)	Cycle number
Pre-denaturation	94	2	1
Denaturation	94	1	36
Annealing	49	1	36
Elongation	72	2	36
Last elongation	72	5	1
Storage	4	∞	

2.5. Antagonistic Effects of Isolates Against Pathogens

Biomass was removed by centrifugation after isolates were grown in TSB medium. Then, the effect of culture fluids against pathogenic microorganisms was investigated using the disc diffusion method. Gram positive and Gram negative organisms were spread on the surfaces of Mueller Hinton agar media. Discs (diameter, 5 mm) were placed on the surface of each plate. Then centrifuged supernatant of AO1-AO4 loaded on discs. The plates were incubated at 37 °C for 24 hrs [15]. The antagonistic effects of the test strains were determined by measuring the zone diameters formed.

3. RESULTS AND DISCUSSION

The most crucial problem in the treatment of infectious diseases is presence of antibiotic-resistant microorganisms due to widespread use of antibiotics in world. For this reason, in recent years, it has become important to search for new microorganisms that produce powerful antibiotics [16, 17]. *Streptomyces* are source of most antibiotics in the world and in this regard they represent a very important bacterial strain. They are found in almost all environments, from deep sea to high mountains [18-20]. In this study, it was found that three of four bacteria producing anti-microbial substances belong to the genus *Streptomyces*.

3.1. Conventional Analysis of Test Isolates

Four bacteria producing antimicrobial substances were isolated from soil samples collected from different points of Palandöken Mountain. These strains were encoded AO1, AO2, AO3 and AO4. As a result of the analysis, all isolates are catalase positive, and AO1, AO2, AO3 are oxidase negative but AO4 is oxidase positive. Details of conventional tests are given in Table 2.

Table 2. Conventional analysis results of isolates

	Oxidase	Catalase	pH	Temp. (°C)	NaCl (%)	Gram
OA1	-	+	5-9	10-45	0-8	+
OA2	-	+	6-8	15-40	0-4	+
OA3	-	+	4-9	10-45	0-6	+
OA4	+	+	6-9	15-40	0-2	-

Zheng et al. reported that *Streptomyces* ambofaciens was oxidase negative and catalase positive [21]. Also, Baltaci et al. stated that *Streptomyces* violaceochromogenes was oxidase negative and catalase positive [11]. Our data match the literature in this aspect.

3.2. Molecular Analysis

For molecular characterization of the isolated bacteria, DNA isolation was first performed and then 16s rRNA gene regions were amplified with PCR. The gel image is shown in Figure 1. The amplified gene regions were cloned into pGEM-T Easy Cloning Vector (Promega, Southampton, UK) and sent to Macrogen Company (Netherlands) for sequence analysis. As a result of the sequence analysis all the isolates contained 1409–1426 nucleotides. Later, the sequences were examined by BLAST, GenBank and EzTaxon Tools; results are shown in Table 3. Then, phylogenetic tree of isolated bacteria and standard strains was made using neighbor-joining method (Figure 2).

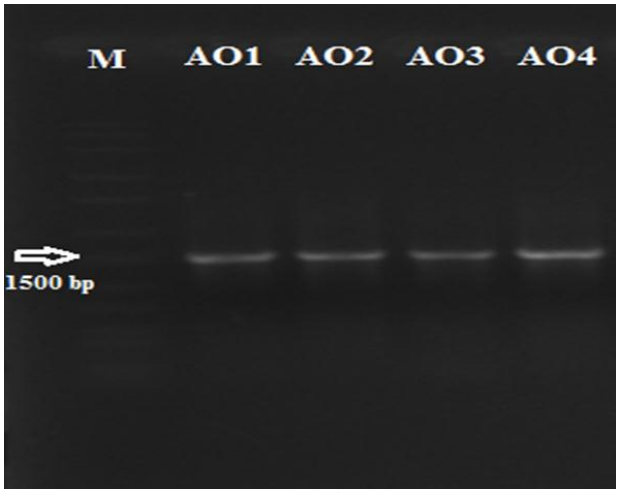

Figure 1. 16S rRNA PCR gel image of isolates

16S rRNA region, which would be substantial in terms of bacterial systematics, was amplified using UNI16S-L: (5'-ATTCTAGAGTTTGATCATGGCTCA-3') and UNI16S-R: (5'- ATGGTACCGTGTGACGGGCGG TGTGTA-3') primers. The primers used amplify a region of about 1500 base pair

Table 3. 16S rRNA gene sequences results and related species

Bacteria strains	Related species	Similarity ratio	Base pair
OA1	*Streptomyces violaceochromogenes*	%99	1425 bp
OA2	*Streptomyces ambofaciens*	%99	1416 bp
OA3	*Streptomyces violaceochromogenes*	%99	1426 bp
OA4	*Sphingomonas melonis*	%99	1409 bp

When the literature data are examined, it is seen that there are many *Streptomyces* species that are isolated from soil and produce antimicrobial substances.

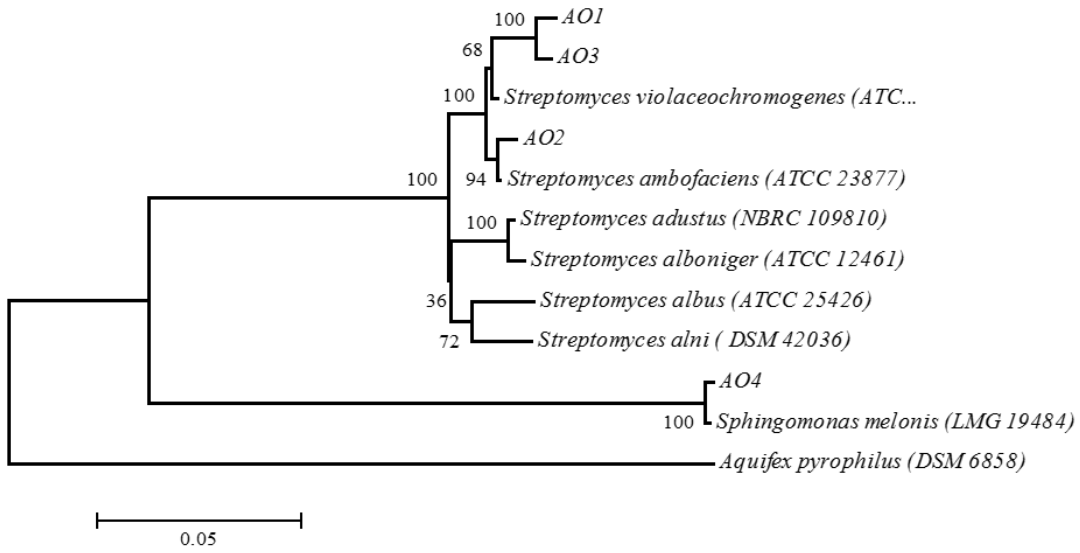

Figure 2. Based on 16S rRNA gene sequence data of the antimicrobial agent producing bacteria isolated from Palandoken montain, the phylogenetic tree was constructed by the neighbor joining method. Aquifex pyrophilus was used as out-group. Bootstrap values based on 1000 replications are listed as percentages at branching points. The scale bar represented 0.05 divergence.

3.3 Determination of Antagonistic Effect

Disc diffusion method was used to determine the antagonistic effect of bacteria against pathogenic microorganisms. Results are given in Table 4.

Table 4. Antagonistic effect of isolates against pathogenic microorganism

Pathogens	OA1	OA2	OA3	OA4
Yersinia pseudotuberculosis	1.8 cm	3 cm	1.4 cm	0.5 cm
Serratia marcescens	2 cm	1.2 cm	2.3 cm	0.25 cm
Klebsiella pneumoniae	2 cm	0.8 cm	2.1 cm	0.55 cm
Streptococcus pyogenes	1 cm	3 cm	0.6 cm	0.6 cm
Staphylococcus epidermidis	1 cm	3 cm	0.7 cm	1.2 cm
Staphylococcus aureus	0 cm	0.1 cm	0.2 cm	0.35 cm
Pseudomonas aeruginosa	1 cm	1 cm	1.6 cm	1.3 cm
Salmonella Typhimurium	0.85 cm	0.3 cm	0.85 cm	1.5 cm
Listeria monocytogenes	1 cm	1 cm	1 cm	0.9 cm
Escherichia coli O157:H7	3 cm	2 cm	3.5 cm	3.2 cm

As a result of disk diffusion analysis, It was determined that most resistant strain against test strains was *Staphylococcus aureus*, and the most sensitive bacteria was *Escherichia coli* O157: H7. There are many studies in literature showing that Staphylococcus aureus are resistant to *Streptomyces* [22-24]. Also many researchers reported that *Escherichia coli* O157: H7 is sensitive to *Streptomyces* [25, 26].

4. CONCLUSION

In conclusion, although the consumption of antibiotics is very common in our country, studies on the production of antibiotics from *Streptomyces* species from which the majority of antibiotics are obtained are quite limited. Turkey is considered as a decent place for isolation of microorganism having ability to produce antimicrobial substances due its unique geographical location and various vegetation.

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